

AD \_\_\_\_\_

Award Number: DAMD17-98-1-8025

TITLE: Role of Estrogen Receptor Target Genes in Breast Cancer

PRINCIPAL INVESTIGATOR: Zhi-Qing Ma, Ph.D.  
Sophia Y. Tsai, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine  
Houston, Texas 77030

REPORT DATE: April 2000

TYPE OF REPORT: **Annual Summary**

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE April 2000		3. REPORT TYPE AND DATES COVERED Annual Summary (1 Apr 99 - 31 Mar 00)	
4. TITLE AND SUBTITLE Role of Estrogen Receptor Target Genes in Breast Cancer				5. FUNDING NUMBERS DAMD17-98-1-8025	
6. AUTHOR(S) Zhi-Qing Ma, Ph.D. Sophia Y. Tsai, Ph.D.					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, Texas 77030  E-MAIL: zma@bcm.tmc.edu				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains colored photographs					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words)  Prolonged exposure of estrogen is closely correlated with the increased incidence of breast cancer. To study the role of estrogen receptor (ER) target genes in the development of breast cancer we construct the regulable repressor KEDPK to directly shutdown the expression of ER target genes. The KEDPK shows a dose-dependent inhibition of ER target genes in transient transfection assays in the presence of its ligand RU486. Stable cell lines expressing KEDPK have been established in both estrogen-dependent (MCF-7) and -independent (LTSD) breast cancer cell lines. Expression of KEDPK inhibits the growth of estrogen-independent cell growth, with no effect on the cell proliferation of estrogen-dependent MCF-7 cells. KEDPK shows 5 to 10% inhibitory effect on the expression of endogenous ER target gene pS2. Currently we have devoted our effort to achieve a better repression of endogenous ER target genes in breast cancer cell lines. The success of developing these repressors will allow us to study the role of ER target genes in breast cancer progression. Results obtained in these studies will be highly relevant to efforts in optimizing the current hormone therapy and gene therapy for breast cancer.					
14. SUBJECT TERMS Breast Cancer				15. NUMBER OF PAGES 26	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited		

20010302 083

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

\_\_\_ Where copyrighted material is quoted, permission has been obtained to use such material.

\_\_\_ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

\_\_\_ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

  
PI - Signature

May 1, 2000  
Date

## Table of Contents

Cover.....	1
SF 298.....	2
Foreword.....	3
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
Appendices.....	8
Legend.....	9

## INTRODUCTION

Breast cancer requires estrogen for its initiation and maintenance before progressing into a more aggressive stage. Estrogen antagonist treatment often results in the arrest or remission of breast cancer growth. However, most of breast cancers recur and become resistant to estrogen ablative therapy. The molecular mechanisms underlying this alteration remain largely undefined. It is well known that biological activity of estrogen is mediated by estrogen receptor (ER). The activated ER will bind to the estrogen response element and ultimately regulates its target gene expression. Thus, the aim of our study is to better understand the role of ER target genes in breast cancer progression. The general approach is to construct a regulable repressor that effectively inhibit the ER target genes expression only in the presence of exogenous ligands. Once the regulable repressor is expressed in tumor cells, the temporal repression of ER target genes can be closely regulated. The success of regulable repressor will have far reaching effect on defining the role of ER target genes in mammary gland ontogenesis and in the recurrence of tumor growth in an estrogen-independent manner.

## BODY

### Generation of regulable repressor of ER target genes

Regulable repressors were generated by linking the KRAB domain (kruppel associated box repressive domain) to the ER DNA binding domain (DBD) and a truncated progesterone receptor ligand binding domain (PR-DBD). We expected that the regulable repressors constructed in this way will dimerize and bind to estrogen responsive element (ERE) to suppress the ER target gene expression in the presence of exogenous ligand RU486 as depicted in **Figure 1**. These regulable repressors have been constructed. Our previous work has shown that the regulable repressor effectively inhibit ER dependent transcription in transient transfection assays only in the presence of exogenous ligand, RU486. The most potent repressor of expression of ER target genes we tested so far is chimeric repressor KEDPK which could effectively inhibit more than 80% of ER-mediated transcription in our transient transfection assay (**Figure 2**). Inhibitory effect appears to be specific to ER target genes, since KEDPK has no effect on glucocorticoid receptor (GR) mediated transcription. Inhibition of ER dependent transcription by KEDPK is dose-dependent (**Figure 3**). Therefore, we have successfully generated a regulable repressor that effectively and specifically inhibits the expression of ER target genes in transient transfection.

### Establishment of estrogen-dependent and -independent breast cancer cell lines stably expressing KEDPK repressor

The repressor KEDPK has been shown to specifically inhibit the ER reporter activity in the presence of RU486, with maximal effect of more than 80% inhibition in transient transfection. We next tried to generate stable cell lines expressing the repressor KEDPK in order to study the role of estrogen receptor target genes in breast cancer cell growth. The repressor KEDPK was subcloned into pcDNA3 expression vector that contains neomycin-resistant gene as selection marker for generation of the stable cell line constitutively expressing KEDPK. The cell lines we chose for stable transfection are estrogen-dependent breast cancer cell line (MCF-7) and estrogen-independent breast cancer cell line (LTSD). LTSD cell lines are kindly provided by Dr. BS Katzenellenbogen's lab which was established by long-term culture of MCF-7 cell line in

the absence of steroids in the effort to study the progression of breast cancer cells from estrogen dependent to independent status. Ten micrograms of pcDNA3-KEDPK plasmid was transfected into MCF-7 and LTSD cell lines. In a parallel experiments, empty plasmid pcDNA3 was transfected into these cell lines as a negative control. Neomycin resistant clones from both lines were selected in the presence of 300 µg/ml neomycin.

Clones resistant to neomycin were isolated from MCF-7 (named MR#) and LTSD (named LR#) cell lines and expanded in the presence of neomycin. Since no antibody against KEDPK is available, the antisense probe of KRAB repression domain was generated to measure the expression of KEDPK at transcriptional level in these clones by RNase protection assay. Representative result of expression of KEDPK in clone LR2-5 is shown in **Figure 4**. The parental cell line LTSD and clone LR2-5 were treated with estrogen, RU486 or estrogen antagonist ICI 164384 as indicated. Twenty-four hours after treatment, RNA samples were isolated and hybridized with the KRAB antisense probe. The antisense cyclophilin probe was included in each sample as a loading control. As shown in **Figure 4**, KEDPK mRNA was detected only in stably transfected LR2-5 clone, not in parental LTSD cell line. The expression of KEDPK was not affected by the different hormonal treatment. Taken together, these data suggested that we established the estrogen-dependent and -independent cells stably expressing KEDPK repressor.

#### **Functional screening of clones stably expressing KEDPK repressor by transient transfection**

The effectiveness of KEDPK on ER target genes in these clones were tested by transient transfection of (ERE)<sub>3</sub>tata Luc reporter. Since no endogenous ER activity was detectable under our assay condition in LTSD cell line, ER expression vector was cotransfected with the reporter gene in functional assay. Representative screening results are shown in **Figure 5**. Addition of estrogen into MR clones stimulated ER dependent transcription. ER activity was clearly inhibited by the repressor KEDPK in the presence of RU486 in these clones (**Figure 5A**). Similar results were observed in the clones isolated from estrogen-independent cell lines LTSD. Estrogen activated ER activity about 10-fold. Addition of exogenous ligand RU486 of KEDPK clearly suppressed the ER mediated luciferase activity (**Figure 5B**). Addition of RU486 had no effect on negative control cell lines transfected with empty expression vector pcDNA3 (data not shown), suggested that the inhibition of ER transcription was mediated by the stably transfected repressor KEDPK. The effectiveness of RU486-dependent inhibition of ER activity varied in different clones. The maximal RU486-dependent inhibition of ER activity in the stable cell lines is lower than that seen in transient transfection. These results indicate that the KEDPK expressed in stable cell lines functions as a RU486-dependent repressor of ER target genes.

#### **Effect of KEDPK on the growth of estrogen-dependent and independent breast cancer cells**

Since we established the estrogen dependent and independent breast cancer cell lines stably expressing KEDPK repressor and the KEDPK could effectively suppress the ER target gene expression in the presence of RU486 in transient transfection., we next tested whether KEDPK could inhibit the breast cancer cell growth dependent or independent of estrogen under these condition. MR3 and LR2-5 cells were cultured and expanded in the presence of 300 µg/ml neomycin. 1000 Cells were plated in 96 well tissue culture plates. Parental cell lines were included as a negative controls. Twenty-four hours later cells were given different treatments as indicated in **Figure 6**. Growth rate of these clones were measured by MTT method on 1, 3 or 5

days after treatments. As expected, estrogen dependent cells grow relatively slowly in the absence of estrogen. Treatment with estrogen moderately increases the growth of both MR3 and its MCF-7 cells, with more stimulating effect on MR3 cells. Under our assay condition, RU486 could slightly increase the cell growth of both MCF-7 and MR3 cells. RU486 has no significant effect on the cell growth of MR3 stimulated by estrogen. In the case of estrogen-independent cell lines, estrogen treatment slightly decreases the LR2-5 cell growth and has no effect on LTSD cell growth. Addition of RU486 slightly increases the growth of LTSD cells. Marginal decreases of LR2-5 cell growth was observed after treatment with RU486, both in the absence or presence of estrogen. The inhibitory effect of KEDPK was quite limited when compared to estrogen antagonist ICI164384 whereby almost no cell growth was observed (data not shown). These results suggested that the repressor KEDPK expressed in stable cell lines is insufficient to significantly inhibit the growth of breast cancer cells either in estrogen-dependent or independent manner.

#### **Effect of KEDPK on endogenous ER responsive genes in breast cancer cell lines**

The ability of KEDPK to suppress endogenous ER responsive genes was evaluated by RNase Protection Assay (RPA) in these breast cancer cell lines. The pS2 gene was used as an ER responsive gene marker to monitor the activity of the repressor KEDPK on endogenous ER target gene expression. RNA samples were isolated from MR3 and LR2-5 cells together with their parental cell lines 24 hrs after various treatments as indicated in **Figure 7**. The antisense probe used in RPA corresponds to the coding region of pS2 and yielded a 180 bp protected fragment. The antisense cyclophilin probe was included in each sample as a reference. Results are shown in **Figure 7A**. In estrogen dependent breast cancer cell line, E2 treatment increased the expression of pS2 in MR3 and its parental cells MCF-7. E2 antagonist ICI effectively blocked the effect of E2 on pS2 gene expression. only 10 % inhibition of E2 induced pS2 expression was observed in the presence of RU486 in MR3 cells. Similar results were observed in estrogen-independent breast cancer cell of LR2-5 (**Figure 7B**). Very little inhibition of ER induced expression of pS2 was observed. Taken together, very limited inhibition of the expression of endogenous ER target gene pS2 were achieved by the repressor KEDPK in the presence of RU486. These results suggest that KEDPK expressed in MR3 and LR2-5 was not sufficient to inhibit the expression of endogenous pS2 ER target gene and cell growth of breast cancer cells in culture.

#### **Ongoing experiments**

It is imperative that the repressor KEDPK suppresses endogenous ER target genes in breast cancer cell lines. Many factors might contribute to the inability of KEDPK to suppress the endogenous ER target genes. It is possible that higher level of KEDPK is required to compete for binding to the promoter of endogenous ER target genes. To get around this problem, we place KEDPK into different expression vectors. KEDPK has been subcloned into episomal mammalian expression vector pCEP4 that has been shown to drive the high expression of recombinant proteins in mammalian cells.

An internal ribosomal entry site (IRES) containing expression vector is also under construction. As depicted in **Figure 8A**, the SV40 promoter will be used to drive the expression of KEDPK and hygromycin-resistant gene linked by IRES, thus translating both genes from a single mRNA. It has been reported that gene expression by cap-independent translation by way of the IRES was not as efficient as cap-dependent translation. After stable transfection, clones



resistant to the hygromycin treatment are supposed to express KEDPK at the level much higher than that by conventional method. We expected that KEDPK expressed at high level could be sufficient to inhibit endogenous ER target genes.

An alternative strategy is to combine KEDPK repression domain with histone-deacetylase (HD1). HD1 is a potent repressor of transcription, presumably by deacetylating histone in the promoter region and thus locking nucleosomes into a tight conformation that precludes access of transcription factors or maintains a suboptimum DNA structure. It is demonstrated in our lab that when fused to the transcriptionally active GAL1-147 DNA-binding domain, HD1 could repress transcriptional activity of a promoter containing GAL4 binding sites. Thus, the improvement to the repressor KEDPK will be made by replacing C-terminal KRAB domain with HD1 as depicted in **Figure 8B**. Once available, the repression activity of modified repressor KDEPH will be test in transient transfection in parallel with KEDPK and eventually in stable cell lines.

## **KEY RESEARCH ACCOMPLISHMENTS**

1. Generated regulable repressor KEDPK of ER target genes.
2. Constructed plasmids transcribing antisense probes of specific ER responsive genes pS2 and complement component 3 (C3) .
3. Established two lines of transgenic mice expressing AIB1 transgene predominantly in mammary glands.

## **REPORTABLE OUTCOMES**

1. Ma ZQ, Tsai MJ, Tsai SY. Suppression of gene expression by tethering KRAB domain to promoter of ER target genes. *J Steroid Biochem Mol Biol* 1999; 69:155-63.
2. Stable MCF-7 breast cancer cell line expressing KEDPK repressor.

## **CONCLUSION**

We have generated a regulable repressor that effectively and significant inhibits the expression of ER target genes in transient transfection in the presence of RU486. Estrogen-dependent and-independent cell lines stably expressing the repressor KEDPK have been established. The repressor was functional active in these stable cell lines as demonstrated in transient transfection. Although the repressor could slightly inhibits the expression of the endogenous ER responsive gene pS2, it is insufficient to block the growth of estrogen-dependent or -independent breast cancer cells in culture. Effort to achieve the maximal inhibition of the endogenous ER target genes will be carried out by expressing high level of the repressor or generating more effective repressors. The role of ER target genes in breast cancer growth will be determined once these cell lines are available.

## **APPENDICES**

1. Legend.
2. Figures.



## Legend

Figure 1. Model of inducible repressor system.

The regulable repressor contains a DNA-binding domain binding to estrogen response element (ERE), a transcription repression domain obtained from Kruppel-Associated Box (KRAB), and a mutated PR ligand-binding domain that responds to antiprogesterin, RU486. The regulable repressor constructed in this way can compete with wild-type ER for ERE binding and turn off all the ER target genes in breast cancer cells in the presence of RU486.

Figure 2. Specific inhibition of ER-dependent activation by the regulable repressor, KEDPK.

Panel A. An (ERE)<sub>3</sub>tata-Luc reporter construct (100 ng) and a human ER expression plasmid (50 ng) was transfected along with or without regulable repressor, KEDPK (200 ng), into HeLa cells using lipofectin. After 6 hr of transfection, cells were washed and incubated in the presence of E<sub>2</sub> (1 nM) for an additional 24 hr with or without RU486 (10 nM), as indicated. The magnitude of ER activation by E<sub>2</sub> alone was set at 100%.

Panel B. HeLa cells were transfected with 50 ng of reporter GREtk-Luc, 50 ng of GR\* (LBD truncation) with or without 100 ng regulable repressor, KEDPK. Luciferase activity was assayed 24 hrs after treatment with or without 100 nM RU486. Luciferase activity was normalized for protein.

A single experiment representative of at least two independent experiments is detailed above. The data shown indicates the mean  $\pm$  SEM of quadruplicate estimations.

Figure 3. Inhibition of ER Activity by KEDPK in a dose-dependent manner.

Panel A. Cells were transfected with (ERE)<sub>3</sub>tata-Luc reporter (100 ng), human ER expression plasmid (50 ng), and an increasing amount of KEDPK repressor construct (50, 100, 200 ng). Cells were treated with 10 nM 17 $\beta$ -estradiol and 100 nM RU486 for 24 hrs as indicated.

Panel B. HeLa cells were transiently transfected with 50 ng of the (ERE)<sub>3</sub>tata-Luc reporter, 50 ng of the human ER expression plasmid, and 200 ng of the repressor construct, KEDPK. Cultures were treated with 17 $\beta$ -estradiol (10 nM) and different concentrations of RU486 for 24 hrs as indicated.

The magnitude of ER activation by E<sub>2</sub> alone was set at 100%. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean  $\pm$  SEM of quadruplicate estimations.

Figure 4. Expression of KEDPK in transfected estrogen-independent breast cancer cell lines

RNA isolated from LR2-5 and parental LTSD cell lines 24 hours after different treatments was isolated and hybridized to KRAB and cyclophilin antisense probes. RNase analysis of KRAB transcripts was carried out according manufacturer's instruction (Ambion). Double protected bands of KRAB shown in autoradiography are caused by minor differences of cloning size of KRAB domain between N-terminal and C-terminal.

Figure 5. Functional screening of estrogen-dependent and -independent breast cancer cell lines stably expressing KEDPK.

Breast cancer cells were transfected with pcDNA3-KEDPK using calcium phosphate precipitation. The transfected cells were selected in the presence of 300  $\mu$ g/ml neomycin. At

their appearance, isolated colonies of neomycin-resistant cells were taken and cultured separately.

Panel A. Clones derived from estrogen-dependent cell line MCF-7 (MR1, MR2 and MR3). 100 ng (ERE)<sub>3</sub>tata-Luc reporter was transfected into the cells.

Panel B. Clones derived from estrogen-independent cell line LTSD (LR2-3 and LR2-5). 25 ng ER expression vector and 100 ng (ERE)<sub>3</sub>tata-Luc reporter was transfected into the cell.

Luciferase activity was determined 24 hrs after 17 $\beta$ -estradiol and RU486 treatment. Results indicate that selected clones respond differently to RU486 in suppression of ER mediated transcription.

Figure 6. Effect of KEDPK on estrogen-dependent and -independent growth of breast cancer cells

Estrogen dependent MCF-7 and MR3 cells are maintained in normal DMEM+10% FCS medium, whereas estrogen independent LTSD and LR2-5 cells are cultured in DMEM+10% stripped FCS medium without phenol red. To measure the rate of cell growth, 1000 cells were plated in 96 well tissue culture plates in DMEM+10% stripped FCS medium without phenol red. 24 hour later, cells were received a different treatments as indicated in the figures. 1, 3 or 5 days after treatment, cells were treated with MTT. O.D. of each well were read in microtiter reader at 540 nm. A single experiment representative of three independent experiments is detailed above. (\*:  $P < 0.05$  compared to control by student  $t$  test,  $n=6$ )

Figure 7. Effect of KEDPK on endogenous ER responsive gene pS2 in breast cancer cell lines

RNA isolated from estrogen-dependent (MCF-7 and MR3) and estrogen-independent (LTSD and LR2-5) breast cancer cell lines LR2-5 and parental LTSD cell lines was isolated 24 hours after different treatments and then hybridized to pS2 antisense probes. Cyclophilin probe was included in each sample as loading control. RNase analysis of pS2 transcripts was carried out according manufacturer's instruction (Ambion). Arrowheads indicate the protected fragments of pS2 and cyclophilin. Autoradiographies of RNase protection analysis were scanned and quantitated as relative optic density (relative O.D.) after normalizing to internal control. Results are drawn in left panels of their corresponding autoradiographies.

Figure 9. Schematic illustrating components of modified vectors.

Panel A: Modified expression vector of KEDPK.

Panel B: Improved regulable repressor.

## Inducible Repressor for ER Target genes

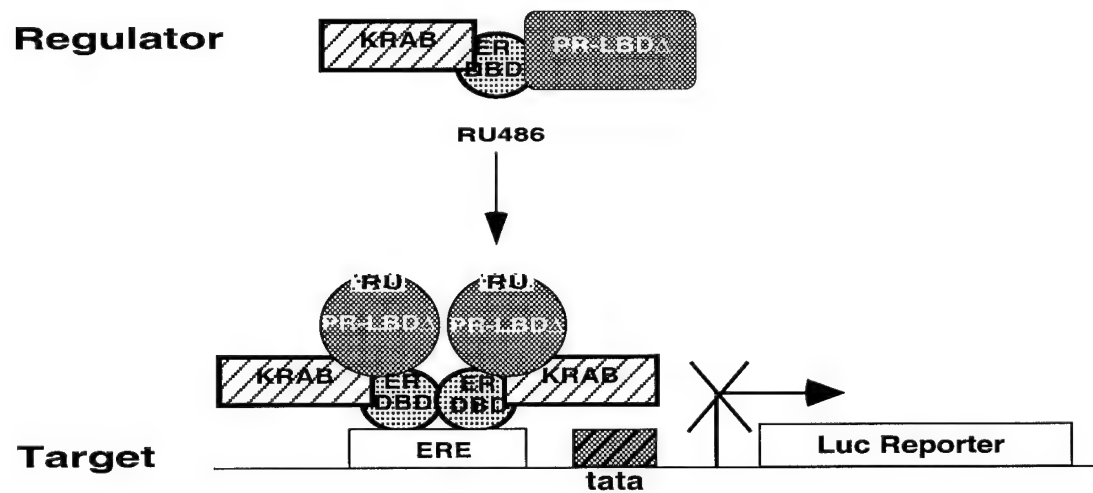
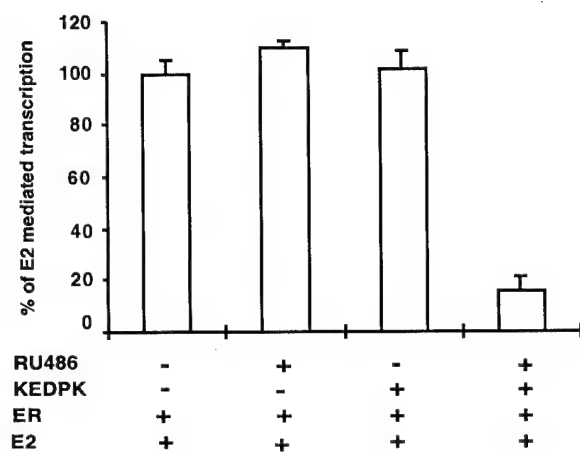


Figure 1

## Specific Inhibition of ER-Dependent Activation by the Regurable Repressor, KEDPK

**A.**



**B.**

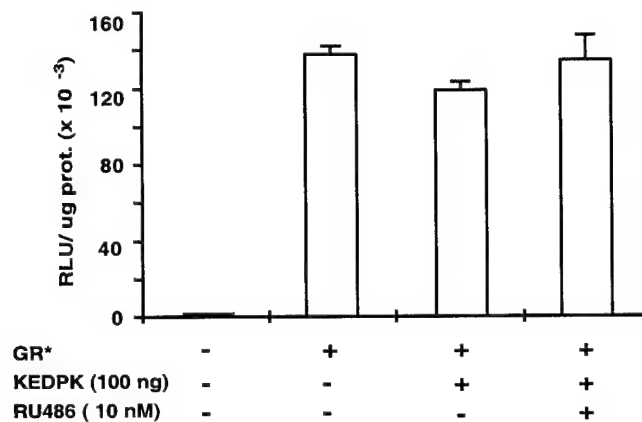
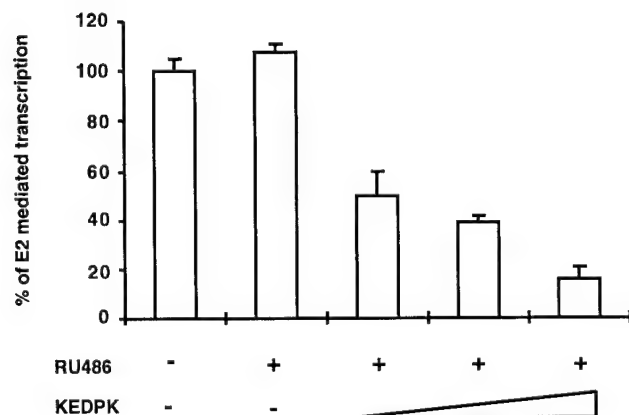


Figure 2

## Inhibition of ER Activity by KEDPK in a Dose-Dependent Manner

**A.**



**B.**

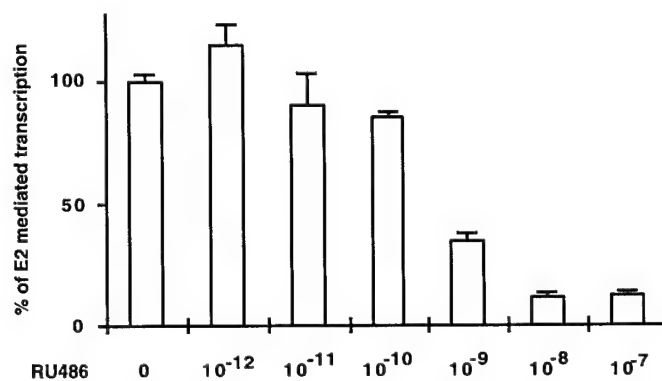


Figure 3

# Expression of KEDPK in Transfected Estrogen-Independent Breast Cancer Cell Lines

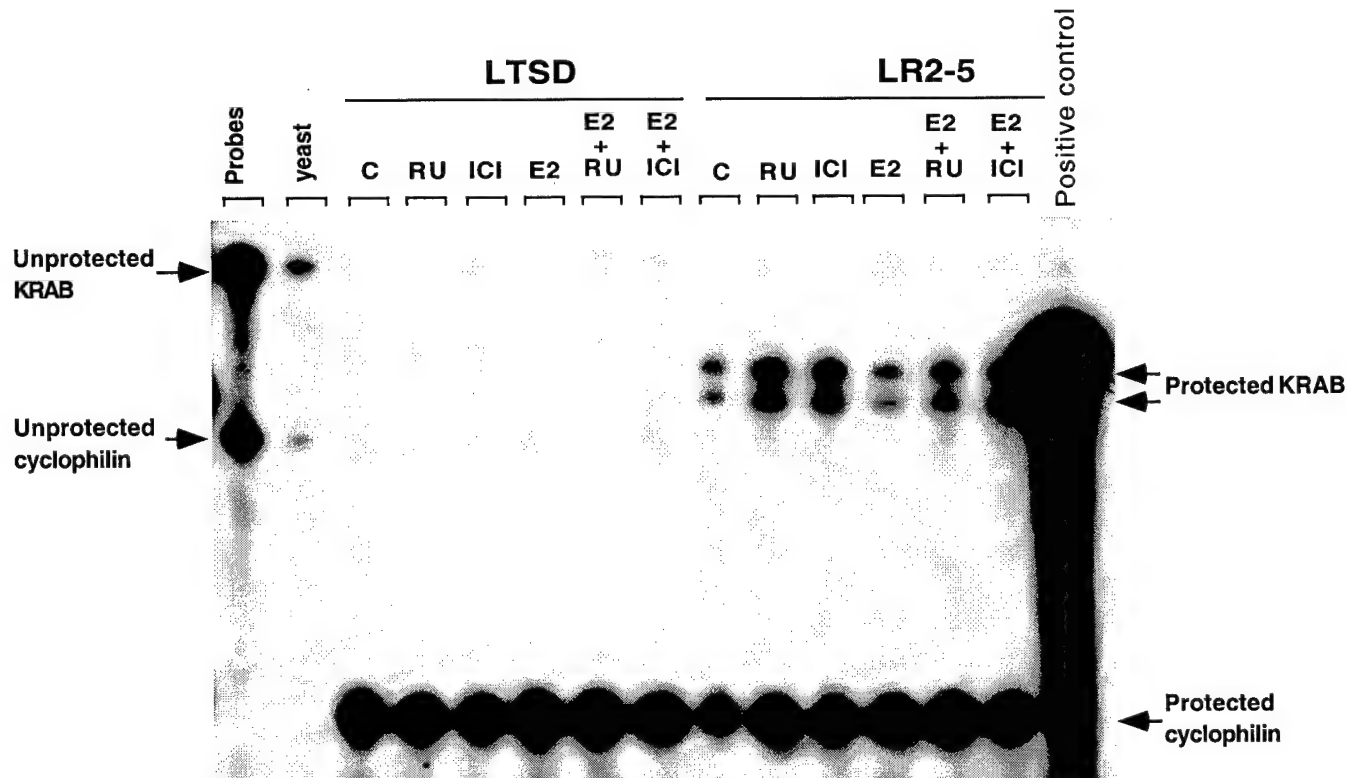
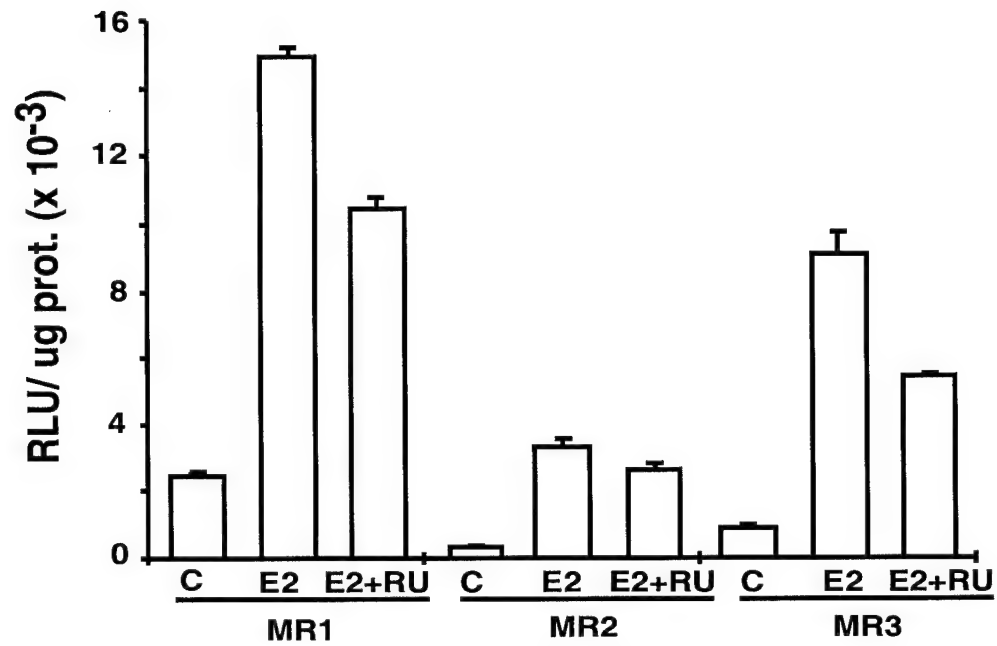


Figure 4

Functional Screening of E2-Dependent and -Independent Breast Cancer Cell Lines Stably Expressing KEDPK

A.



B.

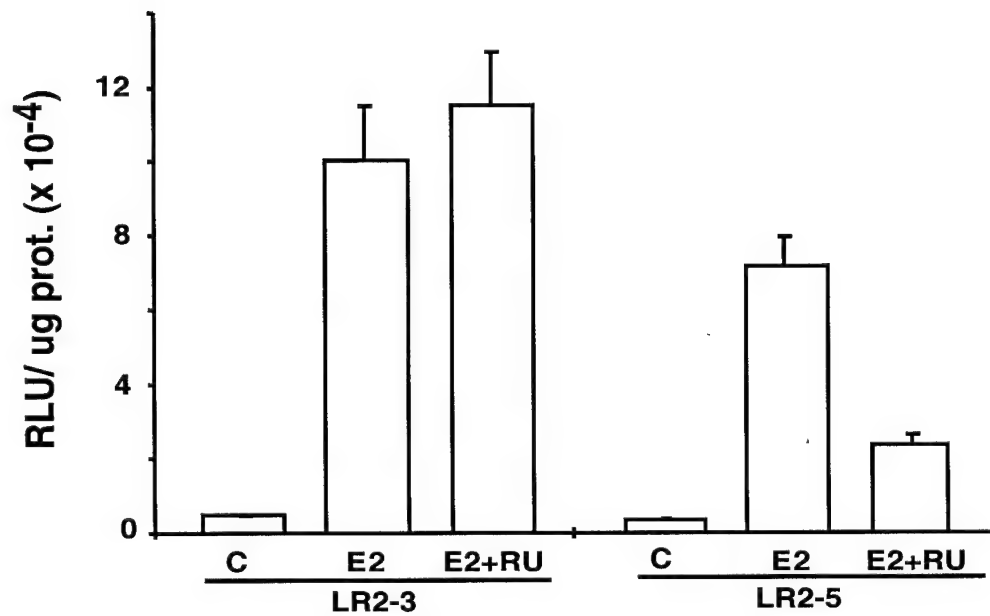


Figure 5

# Effect of KEDPK on Estrogen-Dependent and -Independent Growth of Breast Cancer Cells

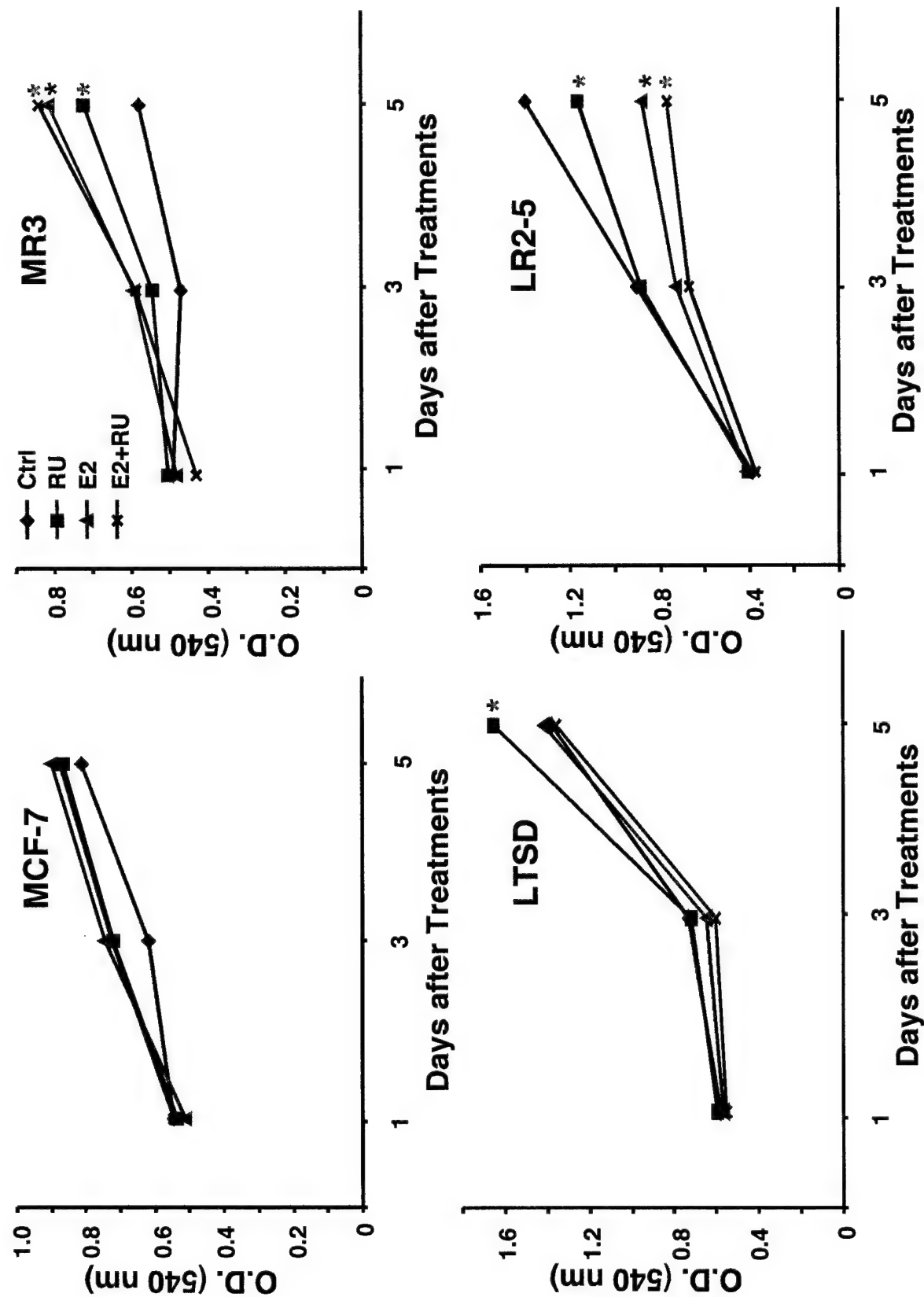
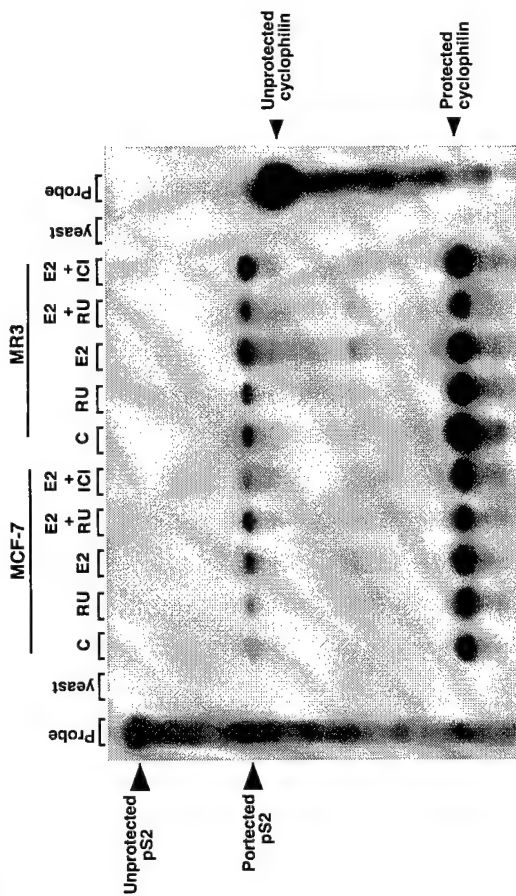


Figure 6



# Effect of KEDPK on Endogenous ER Responsive Gene pS2 in Breast Cancer Cell Lines

A.



B.

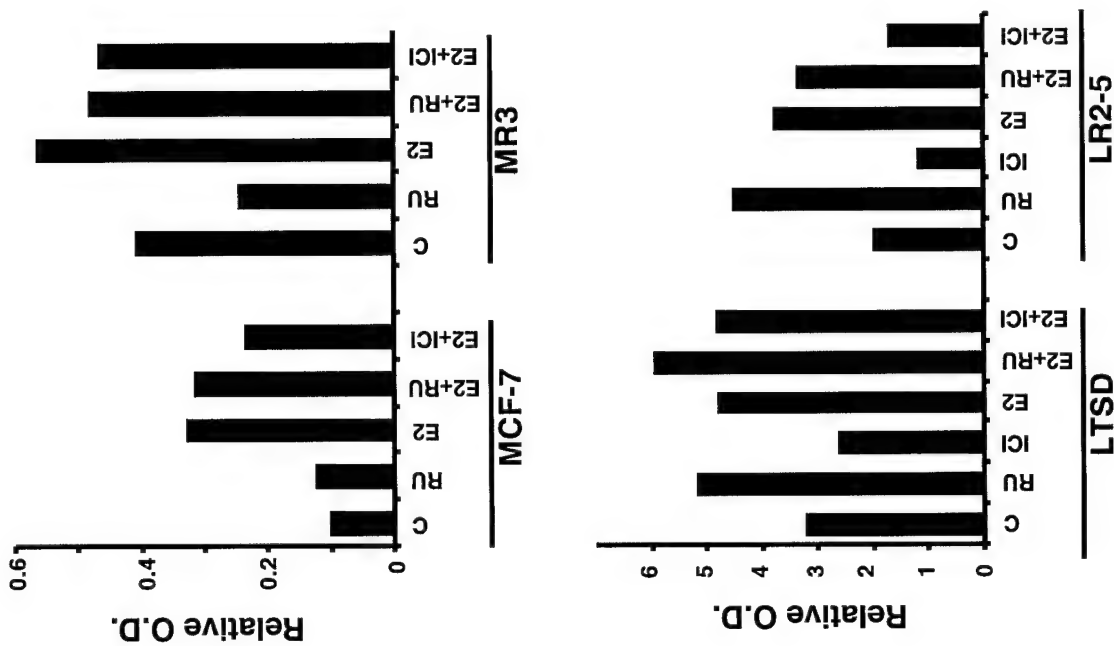
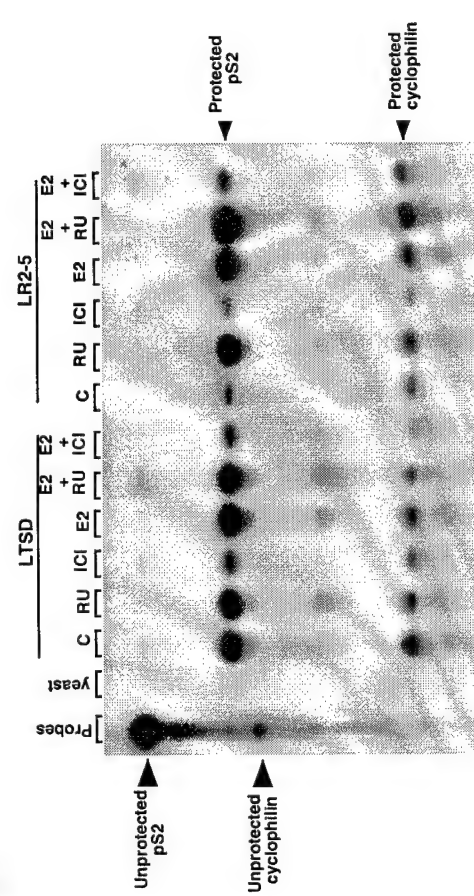
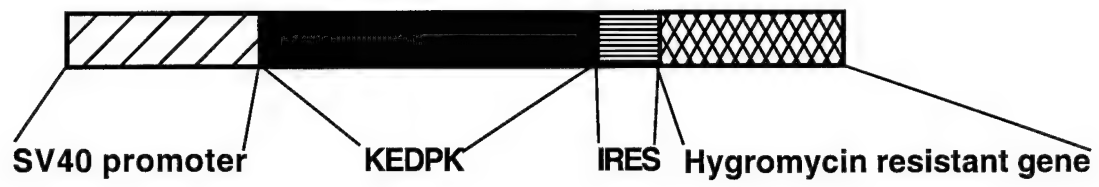
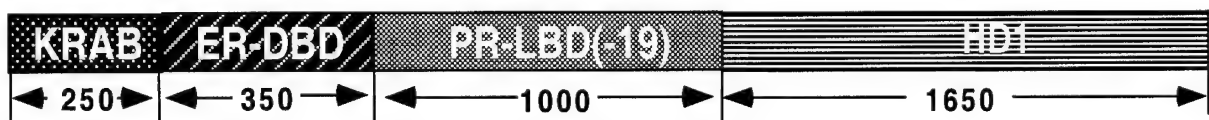


Figure 7

**A.**



**B.**



**Figure 8**



## Suppression of gene expression by tethering KRAB domain to promoter of ER target genes<sup>☆</sup>

Zhi-Qing Ma, Ming-Jer Tsai, Sophia Y. Tsai \*

*Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*

### Abstract

Estrogens play an important role in the development and progression of breast cancer. Although estrogen antagonist treatment often results in the arrest or remission of breast cancer growth, most breast cancers recur and become resistant to estrogen ablative therapy. The molecular mechanisms underlying these actions remain largely undefined. It is hypothesized that tumor cells of an advanced stage may develop compensatory pathways to stimulate the expression of estrogen receptor (ER) target genes or downstream events, independent of estrogen action. In this study, we developed a chimeric repressor to turn off ER target genes with the aim of directly investigating the role of ER target genes in tumor progression. The chimeric repressor contains the ER DNA-binding domain that recognizes estrogen response elements (EREs), a Kruppel-associated box (KRAB) repressor domain which silences target genes when tethered to their promoter regions and a truncated progesterone ligand-binding domain which responds only to the exogenous synthetic ligand, RU486. The ability of the chimeric repressor to block ER mediated transcription was assessed in transient transfection assays. ER-induced reporter activity was inhibited by the repressor in a dose-dependent manner, with the maximum effect of more than 80% reduction. The inhibitory activity of the chimeric repressor was tightly under the control of RU486. Effective suppression by the repressor on the natural promoter of ER target gene, complement factor 3 (C3), was also observed. The inhibitory activity was specific to ER, since the repressor has no effect on other nuclear receptor systems tested. Furthermore, the repressor could inhibit the 4-hydroxy-tamoxifen (4OH-T)-induced ER activity. Taken together, our results demonstrate that the inducible repressor we have designed could specifically inhibit ER target gene expression in response to an exogenous synthetic ligand. This repressor will provide a useful tool to study the role of ER target genes in breast cancer progression and it may be potentially useful for gene therapy of breast cancer. © 1999 Elsevier Science Ltd. All rights reserved.

**Keywords:** Estrogen receptor target gene; Regulable repressor; Kruppel associated box; Breast cancer

### 1. Introduction

Estrogens play an important role in the regulation of the development and function of sex organs, including the breast and reproductive tract [1] as well as in the maintenance of bone, liver, fat and cardiovascular functions [2]. In the breast, estrogen promotes epithelial cell proliferation and ductal elongation in normal breast morphogenesis [3]. Dysregulation of estrogenic activities often causes malfunction, and breast cancer development [4]. It is well documented

that cumulative exposure to estrogen is associated with initiation and progression of breast cancer [5, 6]. In addition, there is a strong correlation between reproductive history and the incidence of breast cancer [7]. Endocrine therapies in breast cancer patients could drastically reduce tumor formation and recurrence [8].

Most, if not all, effects of estrogens are mediated by the estrogen receptor (ER). ER belongs to a large family of nuclear receptors that function as ligand-inducible transcription factors. As a feature of nuclear receptors, ER is composed of three major functional domains: a ligand binding domain, a DNA binding domain and a transactivation domain. Upon binding to cognate ligand, ER is activated, dimerizes and binds to specific DNA response elements (EREs) in the regulatory region of target genes, eventually stimulating

<sup>☆</sup> Proceedings of Xth International Congress on Hormonal Steroids, Quebec, Canada, 17–21 June 1998.

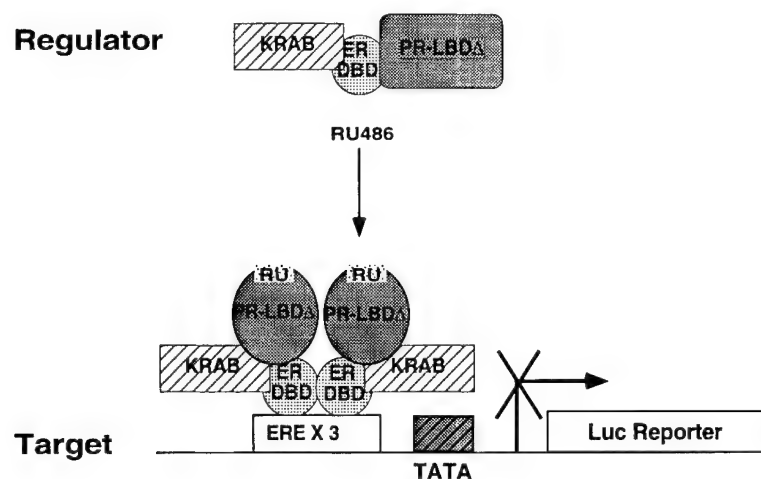
\* Corresponding author. Tel.: 798 6253; fax: 798 8227; e-mail: mtsai@bcm.tmc.edu

target gene expression [9, 10]. Among ER target genes are growth factors which act as direct mitogens in a paracrine or autocrine manner to stimulate epithelial proliferation. More than half of breast carcinomas are found to be ER positive and retain some degree of steroid responsiveness [11]. An increased level of ER expression in breast cancer tissue implies that the tumor could have arisen from a subset of cells, which inappropriately express high levels of ER. This could further lead to the stimulation of cell proliferation and accumulation of genetic mutation, resulting in tumor formation and progression into estrogen independent growth.

The antiestrogen, tamoxifen, has been developed to block the binding of estrogen to its receptor. Tamoxifen has been used successfully to inhibit ER dependent growth of breast cancer [12]. A major unresolved issue is why most breast cancers that contain ER eventually become resistant to estrogen-ablation therapy [13]. It has been proposed that the mutation of ER to a constitutively active receptor or to a recep-

tor which can be activated by estrogen antagonists, like tamoxifen or other steroidal compounds existing in the blood stream may contribute to the transition from estrogen-dependent to -independent tumor growth [14, 15]. If this hypothesis is correct, attempts to block the interaction between ER and estrogen by antagonists will not be able to suppress ER function. However, half of all advanced breast cancers are estrogen receptor positive but resistant to antiestrogen therapy. Some ER negative tumors behave as if they are ER positive in their expression of ER target genes such as the progesterone receptor. Furthermore, many ER mutations identified in tumor cells are also found in healthy cells of breast cancer patients or healthy individuals. Thus, it remains controversial whether ER mutations have a primary role in the transition from estrogen-dependent to -independent states. An additional explanation is that the activation of ER may be possible through ligand independent pathway. Indeed, growth factors, intracellular protein kinases [16-18], the cell cycle regulator cyclin D1 and

A.



B.

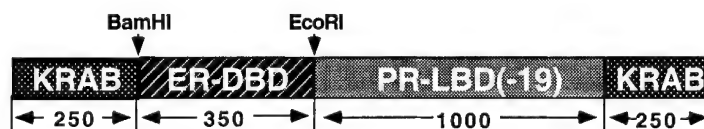


Fig. 1. Inducible repressor system. (A) Model of inducible repressor system. The regulable repressor contains a DNA-binding domain binding to estrogen response element (ERE), a transcription repression domain obtained from Kruppel-associated box (KRAB) and a mutated PR ligand-binding domain which responds to antiprogesterin, RU486. The regulable repressor constructed in this way can compete with wild-type ER for ERE binding and turn off all the ER target genes in breast cancer cells in the presence of RU486. (B) Diagram of regulable repressor KEDPK construct.

cyclin-dependent kinase (cdk2/cyclin A) complex [19–21] have been shown to modulate the activity of unliganded ER in different model systems. It is hypothesized that the unliganded receptor activation occurs via receptor phosphorylation. This phosphorylation leads to changes in receptor conformations whereby the potential to interact with co-activators or other transcription factors may be different from conformations induced by estrogens. This ligand-independent activity may also be enhanced by the addition of the partial agonist tamoxifen.

Though ER could be activated either by its ligand or alternatively by either mutations or ligand-independent pathways, the activated form(s) of ER will ultimately act on the regulatory region of its target genes to exert its biological function. In this study, we constructed a regulable repressor which will bind to an ERE and silence the ER target gene expression in response to exogenous stimuli. A similar strategy has been employed to construct a regulable inducer (GLVP) which activates target gene expression in response to exogenous signal [22,23]. To generate a repressor, we used the Kruppel-associated box (KRAB) which is a highly conserved repression domain in the Kruppel-class zinc finger family of transcription factors [24,25]. When KRAB is linked to a heterologous DNA-binding domain, it can shut off transcription of target genes containing the DNA response element to which the chimeric protein binds [26]. Here we constructed a fusion protein linking KRAB to the estrogen receptor DNA-binding domain to repress all the ER target genes with ERE in their promoters. In order to generate a regulable repressor, we used a truncated ligand binding domain of progesterone receptor which binds specifically to the antiprogesterin, RU486 [27]. Upon binding of RU486, the inducible repressor will dimerize and bind to EREs

to suppress the ER target gene expression as depicted in Fig. 1(A). In this study, we demonstrate that the regulable repressor can inhibit the ER dependent gene expression in response to exogenous added ligand, RU486.

## 2. Materials and methods

### 2.1. Plasmid construction

The KRAB domain and E2F1 DNA binding domain were amplified from pBXG1/Kid-1N (kindly provided by Dr. J. V. Bonventre, Massachusetts General Hospital, Charlestown, MA) and pCMV-E2F1 and ligated together by PCR. The resultant fragment was cut by *Xba*I and *Eco*RI and inserted into MCS sites of pBS-KSII(+) to construct pKS-KE. The truncated PR-LBD(–19)-KRAB fusion fragment was amplified from pCEP4-GL914KRAB [23] by PCR. The PCR product was cut by *Xba*I and *Eco*RI, terminal ends filled by klenow, then ligated into the *Eco*RV site of pKS-KE. The resultant plasmid pKS-KEPK contains the functional KRAB domain at both N- and C-termini of the chimeric construct. E2F1 DNA binding domain was excised with *Bam*HI and *Eco*RI and replaced by a PCR amplified ER DNA-binding domain to generate regulable repressor of ER target genes pKS-KEDPK. The plasmid pKS-KEDPK was then subcloned into the pCMX and pCEP4 (Invitrogen, Carlsbad, CA) expression vectors. Plasmid DNAs were sequenced and checked by *in vitro* transcription/translations. The primers used for PCR amplification (5' primer; 3' primer) are listed as follows:

KRAB: AAGCTTCTAGACTGCAGCTCGAGGCCACCATGGCTCCTGAGCAAAG;

CCGCTTCACGGGATCCTCTCCTTGCTG.

E2F1-DBD: GAGGATCCCGTGAAGCGGAGGCTGGAC;

CCGGAATTCGGAGATCTGAAAGTTCTC.

ER-DBD: CGCGGATCCTATGGAATCTGCCAAGGAG;

CCGGAATTCAGACCCCACTTCACCCCTG.

PR-LBD and KRAB fusion:

CGCGGATCCTTTAAAAAGTTCAATAAAGTCAGAG;

CCGGAATTCTCATCCTTGCTGCAACAGGGAG.

## 2.2. *In vitro* transcription/translation of the regulable repressor KEDPK

1 µg of the KEDPK expression vector was translated *in vitro* in a TNT Coupled Reticulocyte Lysate Systems (Promega) together with an empty expression vector and ER expression vector at 30°C for 30 min. The translated products were then separated in 10% SDS-PAGE gel, fixed, dried and developed following autoradiography.

## 2.3. Gene transfer and luciferase assay

HeLa cells were routinely maintained in Dulbecco's Modified Medium (DMEM; GIBCO, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT). Cells were seeded 24 h before transfection in 6-well tissue culture plates ( $2 \times 10^5$  cells/well) in phenol red-free DMEM containing 10% charcoal/dextran treated FCS. DNA was transiently transfected into cells with lipofectin reagent (GIBCO, Gaithersburg, MD). Cells were transfected for 6 h and then washed with phosphate buffer to remove reagents. Cells were then incubated for an additional 24 h in phenol red-free medium containing 10% charcoal/dextran treated FCS in the absence or presence of hormones. Cell extracts were prepared by adding 300 µl lysis buffer (Promega, Madison, WI) and assayed for luciferase activities (Monolight 2010 Luminometer, Analytical Luminescence Laboratory, MI). All determinations were performed in quadruplicate at least twice.

The reporter plasmid (ERE)<sub>3</sub>TATA-Luc contains 3 copies of the vitellogenin ERE fused upstream of E1A TATA box linked to luciferase (Luc). The C3-Luc reporter, which contains 1.8 kb of the human C3 gene promoter (–1807 to 58) [28], was kindly provided by Jon Rosen (Ligand Pharmaceuticals).

## 3. Results

### 3.1. Generation of an inducible repressor

Based on the modular nature of transcription factors we generated chimeric repressors in attempt to specifically turn off the ER target genes in the presence of exogenous ligand. Regulable repressors contain a KRAB domain either at both N- and C-termini (KEDPK) or only at the C-terminus (EDPK), an ER DNA-binding domain and a truncated progesterone receptor ligand-binding domain (–19) as shown in Fig. 1(B). The KRAB domain we used is a conserved region of 75 amino acids present in the N-terminus of Kid-1, a member of the Kruppel class of transcription factors isolated from rat kidney [29]. Fusion of this

KRAB domain to DNA binding domains of the LacI/ Z, Gal4 or TetR domains has been shown to be able to suppress the expression of respective reporters which contain corresponding binding site in their promoters [24–26]. Here, the KRAB domain was linked to an ER fragment (aa 175 to 282) comprising minimal ER DNA binding domain [30] to make sure that this construct can specifically bind to ERE with affinity compatible to the wild-type ER. The truncated PR ligand binding domain has been found to activate rather than repress reporter activity in the presence of antagonist RU486 [27]. Fusion of this mutated ligand binding domain to heterologous protein has been shown to render it under the control of exogenous ligand RU486 [22, 23]. The specific feature of these regulable repressors is that they should only inhibit the target genes containing an ERE in the presence of RU486.

To ensure that correct proteins are made from the regulable repressor, we performed *in vitro* transcription/translation in parallel with the parental cloning vector and ER expression vector (66 kDa) as controls. As shown in Fig. 2, no protein was translated from the empty vector. The expected size of translated proteins was produced by EDPK and KEDPK expression vectors. These data suggested that the recombinant

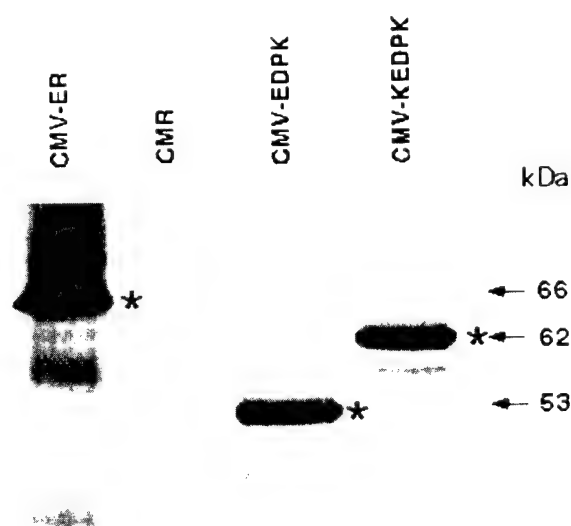


Fig. 2. *In vitro* transcription/translation of the regulable repressor, KEDPK. A TNT coupled reticulocyte lysate system was used to express the KEDPK regulator. An empty expression vector and ER expression vector were included as controls. The translated products were separated in 10% SDS-PAGE gel. \* denotes the expected size of translated proteins.

DNA constructs were able to express the full-length chimeric proteins.

### 3.2. The inducible repressor inhibits ER dependent transcription in the presence of progesterone antagonist, RU486

To test the functional properties of the repressor, HeLa cells were co-transfected with repressor KEDPK and (ERE)<sub>3</sub>TATA-Luc reporter plasmids together with a human ER expression vector. Luciferase activity in response to a saturating dose of E<sub>2</sub> (10<sup>-9</sup> M) and RU486 (10<sup>-8</sup> M) was measured in the absence or presence of repressor plasmids. As shown in Fig. 3(A), there is a seven-fold increase in ER dependent transcription in the presence of E<sub>2</sub>. ER dependent transcription was not affected by addition of RU486. The

chimeric repressor KEDPK has no significant effect on the reporter gene expression in the absence of exogenous ligand, RU486. However, KEDPK could effectively inhibit more than 80% of ER-mediated transcription in the presence of RU486 (10 nM). Thus, KEDPK could compete effectively for the ERE binding site with wild-type ER to suppress the transcription. EDPK could also inhibit ER induced transcription but was less potent than that of KEDPK (data not shown).

The specificity of repressor activity was assessed by co-transfection with reporter plasmid containing glucocorticoid response element (GREtk-Luc) which is palindromic DNA sequences similar to the ERE. Since RU486 has been reported to have glucocorticoid receptor (GR) antagonistic property, a constitutively active form of GR (GR\*) with ligand binding domain trun-

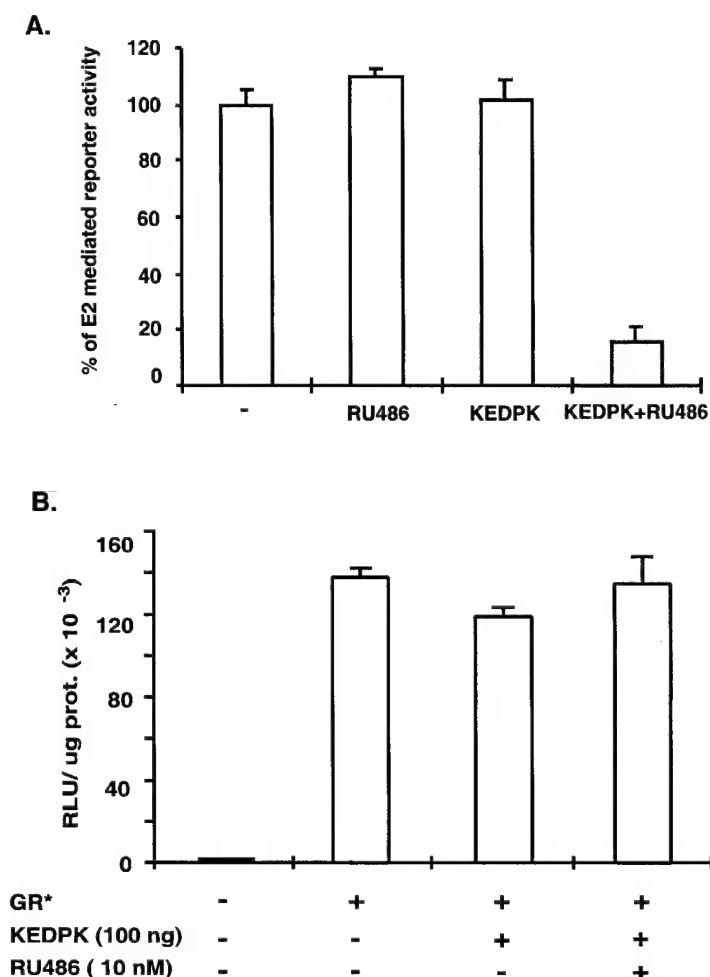


Fig. 3. Specific inhibition of ER-dependent activation by the regulable repressor, KEDPK. Panel A: an (ERE)<sub>3</sub>TATA-Luc reporter construct (100 ng) and a human ER expression plasmid (50 ng) was transfected along with or without regulable repressor, KEDPK (200 ng), into HeLa cells using lipofectin. After 6 h of transfection, cells were washed and incubated in the presence of E<sub>2</sub> (1 nM) for an additional 24 h with or without RU486 (10 nM), as indicated. The magnitude of ER activation by E<sub>2</sub> alone was set at 100%. Panel B: HeLa cells were transfected with 50 ng of reporter GREtk-Luc, 50 ng of GR\* (LBD truncation) with or without 100 ng regulable repressor, KEDPK. Luciferase activity was assayed 24 h after treatment with or without 10 nM RU486. Luciferase activity was normalized for protein quantitation. A single experiment representative of at least two independent experiments is detailed above. The data shown indicates the mean  $\pm$  SEM of quadruplicate estimations.



cation was used to exclude the effect of RU486 on GR activity [31]. The truncated GR (GR\*) stimulated GR reporter gene expression over 50-fold. The repressor has no effect on the GR\* induced transcription either in the absence or in the presence of RU486 [Fig. 3(B)]. Thus, the repressor specifically inhibited ER mediated transcription in transient transfection assays.

### 3.3. Dose-dependent inhibition of ER-mediated transcription by the inducible repressor

To further characterize the potency of the repressor on ER-dependent transcription, different amounts of KEDPK plasmid were co-transfected with the ER expression vector. Results shown in Fig. 4 indicate that inhibitory effect of the repressor on ER transcription was dose-dependent. A 50% reduction of ER-mediated transcription was observed when equal amounts of ER and repressor KEDPK plasmids were cotransfected. Maximum inhibition of the ER mediated transcription was observed when repressor was in 4-fold excess of ER. Further increase in the amount of repressor has an inhibitory effect on the reporter even in the absence of RU486 (data not shown), implying that the excess repressor is binding to the ERE site of the reporter gene in the absence of exogenous added ligand. Next, we assessed the effective dose of RU486 in inducing the repressor activity. As shown in Fig. 5, the repressor exhibited a RU486 dose-dependent regulation of suppressive activity. The maximum inhibition of ER mediated transcription appeared at concentration of 10 nM, which is below the concentration of RU486

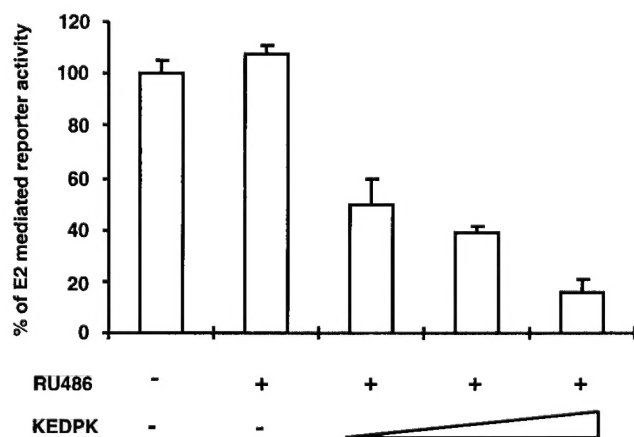


Fig. 4. Inhibition of ER activity by KEDPK in a dose-dependent manner. Cells were transfected with (ERE)<sub>3</sub>TATA-Luc reporter (100 ng), human ER expression plasmid (50 ng) and an increasing amount of KEDPK repressor construct (50, 100, 200 ng). Cells were treated with 10 nM 17 $\beta$ -estradiol and 100 nM RU486 for 24 h as indicated. The magnitude of ER activation by E<sub>2</sub> alone was set at 100%. A single experiment representative of three independent experiments is detailed above. The data shown indicates the mean  $\pm$  SEM of quadruplicate estimations.

required to antagonize any progesterone and glucocorticoid activity. Similar results were observed by co-transfection of repressor and reporter plasmids into the breast cancer cell line MCF-7 (data not shown). These results suggested that RU486 could be used as ligand to regulate KEDPK repressor activity with minimal effects on other steroid hormones.

### 3.4. Inducible repressor that antagonizes ER mediated transcription is independent of cellular and promoter context

ER contains two transactivation domains, AF1 and AF2, which operate in a cell and promoter-specific manner to mediated ER action. Tamoxifen, the most widely used agent in endocrine therapy of breast cancer, acts as a partial agonist of ER in a cell type specific manner. The partial agonist activity of tamoxifen has been proposed to relate to its ability to activate the AF1 of ER [32]. To examine whether the repressor we developed is capable of inhibiting tamoxifen-activated transcription, the repressor plasmid and a (ERE)<sub>3</sub>TATA-Luc reporter were transfected together with an ER expression vector into HepG2 (human hepatocellular carcinoma) cells where AF1 activity of ER was proven to be dominant [33]. As shown in Fig. 6, 4-hydroxy-tamoxifen (4OH-T) treatment of HepG2 cells resulted in an 11-fold induction of ER mediated transcription, which is about 10% of the response elicited by E<sub>2</sub>. The repressor KEDPK inhibited the 4HOT-induced ER activity in the presence of RU486 as efficiently as it inhibited E<sub>2</sub> induced activity.

In addition, the efficacy of repressor function was examined on a natural estrogen responsive promoter. In this case we chose the estrogen responsive complement factor 3 (C3) promoter which contains putative ERE [34]. The E<sub>2</sub> was able to stimulate luciferase reporter expression from the natural C3 promoter (Fig. 7). Transcription was almost completely blocked in cells transfected with repressor after treatment of RU486 (10 nM). Taken together, these results indicated that repressor KEDPK could block the ER activity independent of cellular and promoter context.

## 4. Discussion

Nuclear hormone receptors are inducible transcription factors that regulate gene expression in response to their cognate ligands. They usually contain a transactivation or repression domains, a DNA binding domain and a ligand binding domain. Upon ligand binding, nuclear hormone receptors selectively bind through their DNA binding domains to respective response elements in the promoters and enhancers of target genes and directly interact with transcription

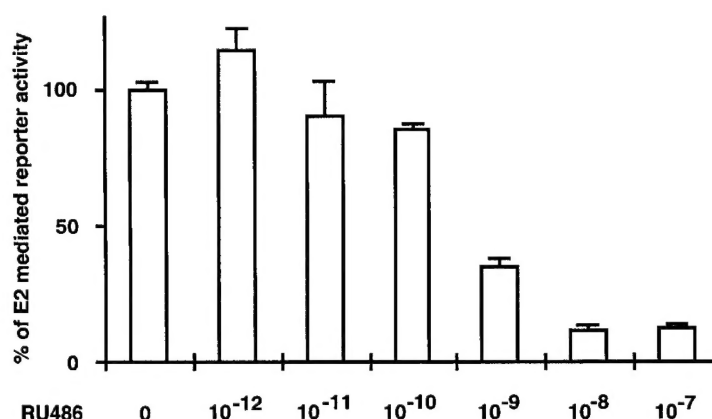


Fig. 5. RU486 dose-dependent curve on KEDPK inhibition of ER activity. HeLa cells were transiently transfected with 50 ng of the (ERE)<sub>3</sub>TATA-Luc reporter, 50 ng of the human ER expression plasmid and 200 ng of the repressor construct, KEDPK. Cultures were treated with 17 $\beta$ -estradiol (10 nM) and different concentrations of RU486 for 24 h as indicated. The magnitude of ER activation by E<sub>2</sub> alone was set at 100%. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean  $\pm$  SEM of quadruplicate estimations.

initiation complexes, coactivators or corepressors to activate or repress transcription of their target genes [35]. By taking advantage of the modular nature of nuclear hormone receptors, several regulable gene expression systems have been developed to express exogenous gene products [23,36,37]. The basic strategy of these regulable systems relies on two components: a chimeric transactivator genetically engineered from bacterial repressor proteins or eukaryotic receptors to function as a gene switch in response to exogenous ligands and a corresponding heterogeneous promoter, whose activity is under the control of the chimeric transactivator. By linking the gene of interest to a response element of the chimeric transactivators, the ex-

pression of particular gene can be regulated using exogenous ligand. These regulable systems have been used in studying the function of specific protein *in vivo* and in producing therapeutic proteins for the treatment of human disease through gene therapy.

In this paper, we converted the transactivator to a repressor as a means to repress the endogenous gene expression in a regulable fashion. A number of transcription repressors have been characterized that negatively regulate specific genes transcribed by RNA polymerase II. Analyses of the mechanisms of transcription repression have revealed distinct classes of repression including steric hindrance of transcriptional activators from DNA binding, direct interaction with basal transcription machinery or squelching a specific activator proteins. Like transcriptional activators, many transcriptional repressors are modular factors. One of the best characterized repressor domains, KRAB, can be separated from DNA binding domain. It has been shown that when fused to a heterogeneous DNA binding domain, KRAB suppresses the activating function of various defined transcription activators [26].

In the present study, we used the KRAB domain to create a chimeric transcription repressor directly targeted to the endogenous promoter of a gene of interest. The endogenous target genes we chose are ER target genes. It is well known that ER can regulate its target gene expression by binding to the specific DNA sequences known as estrogen response elements (EREs). The constructed repressor is supposed to effectively inhibit the expression of ER target genes by competing for ERE binding sites with ER and directly interacting with basal transcription factors to interfere with RNA polymerase II-mediated transcription. ER target genes, such as pS2 [38] and C3 [34] have been

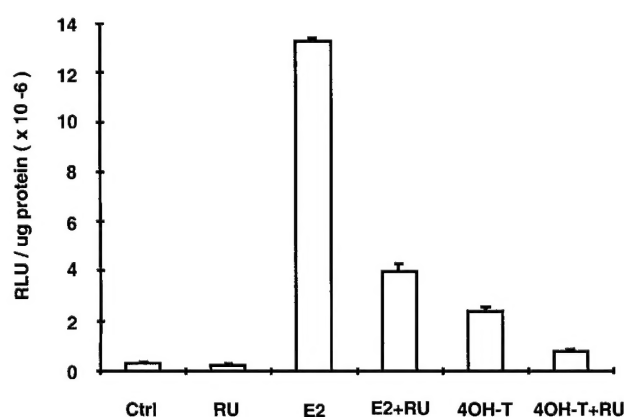


Fig. 6. The effect of KEDPK on 4-hydroxy-tamoxifen (4OH-T) stimulated ER activity. The repressor plasmid (200 ng) and (ERE)<sub>3</sub>TATA-Luc reporter (100 ng) were transfected together with an ER expression vector (100 ng) into HepG2 (human hepatocellular carcinoma) cells. Luciferase activity was normalized for quantity of protein. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean  $\pm$  SEM of quadruplicate estimations.

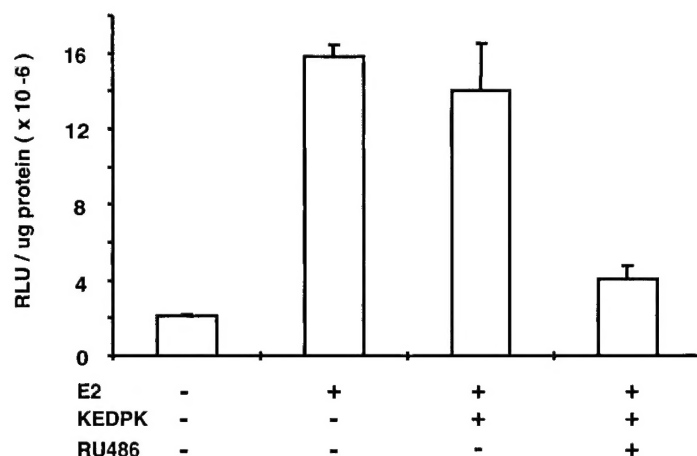


Fig. 7. The effect of KEDPK on ER mediated natural promoter activity of complement factor 3 (C3). HeLa cells were transfected with C3-Luc promoter (100 ng), ER expression plasmid (100 ng) and KEDPK repressor construct (200 ng). Cells were treated with 10 nM 17 $\beta$ -estradiol and 100 nM RU486 for 24 h as indicated. Luciferase activity was normalized for quantity of protein. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean  $\pm$  SEM of quadruplicate estimations.

identified and found to contain conserved palindromic EREs in their promoter regions, which provide reliable markers to examine the efficiency of regulable repressor of ER target genes. Since the roles of ER and its target genes in the development of hormone-dependent or -independent breast cancers have not been determined, the designed repressor can bypass ER to block the expression of ER target genes. In this way, we should be able to examine directly the role of ER target genes in breast cancer progression.

The repressor we constructed was shown to effectively inhibit ER mediated transcription in transient transfection assay. More than 80% inhibition of ER activity could be achieved by increasing the amount of repressor plasmid. The inhibitory activity of the repressor was shown to be tightly controlled by exogenous ligand, RU486, with the maximum effect at 10 nM. This result is similar to the concentration observed in the transactivation system (GLVP) developed in this lab [22]. No effect of repressor on ERE promoter was observed in the absence of ligand, suggesting that ligand binding domain could control repressor dimerization and binding to the specific DNA sequence. Since the repressor specifically inhibits ER mediated transcription, it is unlikely that the suppressive effect is due to the squelching of specific activator protein. Most likely, the inducible repressor dimerizes and competes for DNA binding sites with ER in the presence of RU486. Furthermore, by tethering KRAB to the promoter, it can then carry out its repressive function through interacting with basal transcription machinery and silencing the promoter activity.

Taken together, the chimeric repressor, we designed, is effective and specific in inhibiting ER target gene expression in response to exogenous ligand. Therefore, this repressor will provide a useful tool for studying

the role of ER target genes in breast cancer progression and be potentially useful for gene therapy of breast cancer.

#### Acknowledgements

We thank Dr Jon Rosen and Dr J. V. Bonventre for providing plasmids used in this study. This work was supported by the U.S. Army Medical Research and Material Command DAMD-17-94-J-4400 to S. Y. T., DAMD-17-96-1-6155 to M. J. T. and postdoctoral fellowship (DAMD-17-98-1-8025) to Z. Q. M.

#### References

- [1] D.W. Pfaff, S. Schwartz-Giblin, Cellular mechanisms of female reproductive behaviors, in: E. Knobil, J. D. Neil, L. L. Ewing, G. S. Greenwald, C. L. Market and D. W. Pfaff (Eds.), *The Physiology of Reproduction*, Vol. 2. Raven Press, New York, 1988, pp. 1487–1568.
- [2] D.R. Ciocca, L.M. Roig, Estrogen receptors in human nontarget tissues: biological and clinical implications, *Endocrine Reviews* 16 (1995) 35–62.
- [3] J.L. Ferdick, A.M. Raafat, S.Z. Haslam, Mammary gland growth and development from the postnatal period to postmenopause: ovarian steroid receptor ontogeny and regulation in the mouse, *Journal of Mammary Gland Biology and Neoplasia* 3 (1998) 7–22.
- [4] I.H. Russo, J. Russo, Role of hormones in mammary cancer initiation and progression, *Journal of Mammary Gland Biology and Neoplasia* 3 (1998) 49–61.
- [5] M.C. Pike, M.D. Krailo, B.E. Henderson, J.T. Casagrande, D.G. Hoel, 'Hormonal' risk factors, 'breast tissue age' and the age-incidence of breast cancer, *Nature* 303 (1984) 767–770.
- [6] B.E. Henderson, R. Ross, L. Bernstein, Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture, *Cancer Research* 48 (1988) 246–253.

- [7] J.L. Kelsey, M.D. Gammon, The epidemiology of breast cancer, *Ca: a Cancer Journal for Clinicians* 41 (1991) 146–165.
- [8] P.E. Lonning, E.A. Lien, Mechanisms of action of endocrine treatment in breast cancer, *Critical Reviews in Oncology/Hematology* 21 (1995) 158–193.
- [9] B.W. O'Malley, The steroid receptor superfamily: more excitement predicted for the future, *Molecular Endocrinology* 4 (1990) 363–369.
- [10] M.J. Tsai, B.W. O'Malley, Molecular mechanisms of action of steroid/thyroid receptor superfamily members, *Annual Review of Biochemistry* 63 (1994) 451–486.
- [11] M.E. Lippman, J.C. Allegra, Quantitative estrogen receptor analysis: the response to endocrine and cytotoxic chemotherapy in human breast cancer and the disease-free interval, *Cancer* 46 (1980) 2829–2843.
- [12] V.C. Jordan, Overview from an international conference on long-term tamoxifen therapy for breast cancer, *Journal of National Cancer Institute* 84 (1992) 231–234.
- [13] K. Horwitz, How do breast cancer become hormone resistant, *Journal of Steroid Biochemistry and Molecular Biology* 49 (1994) 295–302.
- [14] P. Lemieux, S. Fuqua, The role of the estrogen receptor in tumor progression, *Journal of Steroid Biochemistry and Molecular Biology* 56 (1996) 87–91.
- [15] L.C. Murphy, H. Dotzlaw, E. Leygue, D. Douglas, A. Coutts, P.H. Watson, Estrogen receptor variants and mutations, *Journal of Steroid Biochemistry and Molecular Biology* 62 (1997) 363–372.
- [16] R.F. Power, S.K. Mani, J. Codina, O.M. Conneely, B.W. O'Malley, Dopaminergic and ligand-independent activation of steroid receptors by a novel alternative pathway, *Science* 254 (1991) 1636–1639.
- [17] H. Cho, B.S. Katzenellenbogen, Synergistic activation of estrogen receptor-mediated transcription by estradiol and protein kinase activators, *Molecular Endocrinology* 7 (1993) 442–452.
- [18] S. Kato, H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, S. Masushige, Y. Gotoh, E. Nishida, H. Kawashima et al, Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase, *Science* 270 (1995) 1491–1494.
- [19] R.M. Zwijsen, E. Wientjens, R. Klompmaker, J. van der Sman, R. Bernards, R.J. Michalides, CDK-independent activation of estrogen receptor by cyclin D1, *Cell* 88 (1997) 405–415.
- [20] E. Neuman, M.H. Ladha, N. Lin, T.M. Upton, S.J. Miller, J. DiRenzo, R.G. Pestell, P.W. Hinds, S.F. Dowdy, M. Brown, M.E. Ewen, Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4, *Molecular and Cellular Biology* 17 (1997) 5338–5347.
- [21] J.M. Trowbridge, I. Rogatsky, M.J. Garabedian, Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex, *Proceedings of the National Academy of Sciences of the United States of America* 94 (1997) 10132–10137.
- [22] Y. Wang, B.W.J. O'Malley, S.Y. Tsai, B.W. O'Malley, A regulatory system for use in gene transfer, *Proceedings of the National Academy of Sciences of the United States of America* 91 (1994) 8180–8184.
- [23] Y. Wang, F.J. DeMayo, S.Y. Tsai, B.W. O'Malley, Ligand-inducible and liver-specific target gene expression in transgenic mice, *Nature Biotechnology* 15 (1997) 239–243.
- [24] J.F. Margolin, J.R. Friedman, W.K. Meyer, H. Vissing, H.J. Thiesen, F.J.R. Rauscher, Kruppel-associated boxes are potent transcriptional repression domains, *Proceedings of the National Academy of Sciences of the United States of America* 91 (1994) 4509–4513.
- [25] R. Witzgall, E. O'Leary, A. Leaf, D. Onaldi, J.V. Bonventre, The Kruppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression, *Proceedings of the National Academy of Sciences of the United States of America* 91 (1994) 4514–4518.
- [26] G. Pengue, L. Lania, Kruppel-associated box-mediated repression of RNA polymerase II promoters is influenced by the arrangement of basal promoter elements, *Proceedings of the National Academy of Sciences of the United States of America* 93 (1996) 1015–1020.
- [27] E. Vegeto, G.F. Allan, W.T. Schrader, M.J. Tsai, D.P. McDonnell, B.W. O'Malley, The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor, *Cell* 69 (1992) 703–713.
- [28] D.P. Vik, P. Amiguet, G.J. Moffat, M. Fey, F. Amiguet-Barras, R.A. Wetsel, B.F. Tack, Structural features of the human C3 gene: intron/exon organization, transcriptional start site, and promoter region sequence, *Biochemistry* 30 (1991) 1080–1085.
- [29] R. Witzgall, E. O'Leary, R. Gessner, A.J. Ouellette, J.V. Bonventre, Kid-1, a putative renal transcription factor: regulation during ontogeny and in response to ischemia and toxic injury, *Molecular and Cellular Biology* 13 (1993) 1933–1942.
- [30] S. Mader, P. Chambon, J.H. White, Defining a minimal estrogen receptor DNA binding domain, *Nucleic Acids Research* 21 (1993) 1125–1132.
- [31] S. Rusconi, K.R. Yamamoto, Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor, *EMBO Journal* 6 (1987) 1309–1315.
- [32] M. Berry, D. Metzger, P. Chambon, Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen, *EMBO Journal* 9 (1990) 2811–2818.
- [33] M.T. Tzukerman, A. Esty, D. Santiso-Mere, P. Danielian, M.G. Parker, R.B. Stein, J.W. Pike, D.P. McDonnell, Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions, *Molecular Endocrinology* 8 (1994) 21–30.
- [34] Z. Nawaz, M.J. Tsai, D.P. McDonnell, B.W. O'Malley, Identification of novel steroid-response elements, *Gene Expression* 2 (1992) 39–47.
- [35] H. Shibata, T.E. Spencer, S.A. Onate, G. Jenster, S.Y. Tsai, M.-J. Tsai, B.W. O'Malley, Role of coactivators and corepressors in the mechanism of steroid/thyroid receptor action, *Recent Progress in Hormone Research* 52 (1997) 141–165.
- [36] D. No, T.P. Yao, R.M. Evans, Ecdysone-inducible gene expression in mammalian cells and transgenic mice, *Proceedings of the National Academy of Sciences of the United States of America* 93 (1996) 3346–3351.
- [37] V.M. Rivera, T. Clackson, S. Natesan, R. Pollock, J.F. Amara, T. Keenan, S.R. Magari, T. Phillips, N.L. Courage, F. Cerasoli Jr., D.A. Holt, M. Gilman, A humanized system for pharmacologic control of gene expression, *Nature Medicine* 2 (1996) 1028–1032 (see comments).
- [38] A.M. Brown, J.M. Jeltsch, M. Roberts, P. Chambon, Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7, *Proceedings of the National Academy of Sciences of the United States of America* 81 (1984) 6344–6348.